Development of a Database of Toxicological Endpoints and Key Characteristics of 86 Agents Known to Cause Cancer in Humans

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Introduction

The majority of the early observations of chemical carcinogenesis linked personal exposures with certain types of cancer (e.g., scrotal cancer in chimney sweeps and lung cancer in cigarette smokers), and only later focused on identification of the causative agents (Loeb and Harris, 2008). The identification of DNA as the genetic material governing fundamental biological processes at the cellular and molecular level, combined with an understanding of the effects of damage to DNA on cell function, provided a key to understanding the mechanisms of human cancer (Carrel et al.1997; Croy et al. 1978). Early descriptors of the mode of action of carcinogens were often based on assays of 'gross'genetic events such as t DNA strand breaks, micronucleus formation, and chromosomal aberrations. Hence, most of the early mechanistic studies focused on the ability of agents to induce genotoxic effects. Recent advances in molecular biology have elucidated the critical role of cellular and molecular processes and pathways (including transcription factors, signaling molecules, and epigenetic apparatus) involved in controlling chemical carcinogenesis. In parallel with breakthroughs in molecular biology, advances in molecular methods such as post-labelling techniques, high throughput microarrays, toxicogenomics and computational systems biology also increased our capacity to identify cancer mechanisms (Krewski et al., 2011).

Molecular and genetic epidemiology has begun to incorporate knowledge gained about the biological mechanisms of human cancer to draw conclusions about individual susceptibility. This has led to understanding of the role of polymorphic gene variants and gene-environment interaction in chemical carcinogenesis. Early concepts of factors affecting inter-individual variation in response to carcinogen exposure were based on research on the effects of the metabolism of carcinogens and on the production of DNA adducts (Perera et al. 1982; Loeb and Harris 2008). More recently, the role of the cellular epigenome, cell signaling, apoptosis, inflammation, immune modulation, and receptor-mediated effects in cancer initiation and promotion have become clearer. At present, the assessment of the mechanisms of cancer induction by different agents considers both the functional and anatomical changes induced by carcinogens, in the context of and a multiple molecular mechanisms of action. This in turn can shed light on the complex interactions among different agents that may increase human cancer risk (Guyton et al. 2009).

Although carcinogenesis is a complex process, common mechanistic characteristics and toxicological endpoints can be identified through an examination of the biological processes demonstrated by established human carcinogens. Birkett et al. (2015) provide an overview of for the mechanistic characteristics of 109 agents identified as causes of human cancer by International Agency for Research on Cancer (IARC) through Volume 109 of the IARC Monographs. A database on mechanistic characteristics of these agents was developed by abstracting mechanistic information from the IARC Monographs, supplemented with a PubMed search to identify additional information that may not have been noted in the Monographs. This database was subsequently used by Krewski et al. (2015) to describe the

key characteristics of human cancer developed by Smith et al. (2015). This chapter describes the construction of the database on key characteristics of human cancer, which was done under the general direction of the IARC Working Group (WG) on 'Tumour-site Concordance and Mechanisms of Carcinogenesis' which convened in Lyon April/November 2012.

The development of the components of the database on key characteristics of human cancer proceeded in two stages. At its initial meeting in April, 2012, the WG developed a list of 24 toxicological endpoints thought to be related to the etiology of human cancer. Data on these toxicological endpoints were later abstracted from the *IARC Monographs*, supplemented by our PubMed search. At the subsequent meeting in November, 2012, the WG developed a list of 10 key characteristics of human cancer, which are described in detail by Smith et al. (2015). The database on key characteristics was then developed by associating the 24 toxicological endpoints with the 10 key characteristics.

In order to populate the IARC cancer mechanisms database, members of our research team (MB working under the direction of RB) examined section 4 of all Monographs to identify information relevant to the 24 toxicological endpoints, in consultation with other members of the team when issues of data interpretation arose. For each Group-1 agent, information on these endpoints was abstracted into the database, with separate entries for human in vivo or in vivo studies and for animal in vivo or in vitro studies. Summary indicators for humans and animals were obtained by combing results across in vivo and vitro sources of information for each agent; similarly, summary indicators for in vivo and in vivo sources were obtained by combining results across human and animal sources of information. Finally, an overall indicator combined across human/animal/in vivo/in vitro sources was derived, with consultation among members of the research team to reach consensus on combined results in cases where this was not immediately obvious.

Using this database on toxicological endpoints expressed by the Group-1 agents, the database on key characteristics demonstrated by human carcinogens was created by associating each of the 24 toxicological endpoints with the most appropriate of the 10 key characteristics. The database of key characteristics was developed for human/animal/in vivo/in vitro sources, with combined results then derived for human and animal studies combined, for in vivo and vitro studies combined, and for human/animal/in vivo/in vitro studies overall.

In addition to examining the *IARC Monographs* as the main sources of mechanistic information, a PubMed search was conducted to supplement the information available in the *Monographs.* The PubMed search was not designed to be a comprehensive systematic review of the scientific literature on the mechanisms of human cancer, but was conducted to check if important mechanistic results may not have been documented in the Monographs, or published after the time of publication of Monographs. Particular attention was paid to recent publications on epigenetics since this area was under developed in Volume 100. The results of quantitative analysis of this mechanistic data base of these IARC carcinogens are presented in another chapter in this volume [Krewski et al this volume).

The present chapter describes the specific toxicological endpoints included in the IARC cancer mechanisms database, and the linkage between these endpoints and the key characteristics. Krewski et al. (2015) subsequently used this database to describe the key

characteristics of Group-1 agents identified through the IARC Monographs Programme. The overarching objective of this project was to develop an enhanced understanding of the mechanistic characteristics of known human carcinogens, and thus to provide a better basis for assessing the cancer risks associated with these agents.

Toxicological Endpoints in Carcinogenesis

Development of the Toxicological Endpoints

At its initial meeting in April 2012, the Working Group identified 24 toxicological endpoints that may be related to cancer induction, including cellular and molecular changes associated with different stages of carcinogenesis. These endpoints are listed in Table 1, along with prototypical assays that may be used to identify agents expressing these endpoints. A description of these 24 endpoints is given below.

[insert Table 1 about here]

1. **DNA damage.** DNA damage is an alteration in the chemical structure or integrity of DNA that includes a break in a strand of DNA, and/or chemical modifications (e.g. covalent binding) of the nucleotide bases. DNA damage is involved in mutagenesis and in the development of cancer (Hoeijmakers, 2009). Several direct and indirect methods have been developed to determine the ability of agents to induce DNA damage. Direct methods include those that detect changes in the chemical structure of DNA (e.g. assays of DNA adducts by ^{32}P-postlabeling techniques), and detection of single strand DNA breaks by Comet assays. Indirect methods examine indicators or biomarkers of DNA and chromosomal damage such as sister chromatid exchange (SCE), unscheduled DNA synthesis (UDS), mitotic recombination and aneuploidy. These effects can be studied in both mammalian and non-mammalian models (e.g., Saccharomyces cerevisiae). Examples of agents that induce DNA damage include chemotherapeutic agents such as busulfan, chlorambucil, methyl-CCNU, and cyclophosphamide (all cause DNA alkylation, and DNA strand breaks); tobacco smoking (causes DNA adducts, single and double strand breaks); ethanol in alcohol beverages (causes chromosomal aberrations, aneuploidy, micronuclei); 4-aminobiphenyl; and benzidine (causes DNA adducts).
2. **Oxidative stress.** Oxidative stress occurs when the antioxidant defenses within a cell are overwhelmed by the production of reactive oxygen species (free radicals), compromising the cell's ability to detoxify the reactive intermediates or to repair the resulting damage (Betteridge, 2000). Examples of assays used to detect this type of effect include those assessing cellular redox state (e.g. measurement of reactive oxygen species (ROS) using DCFH-DA), measurement of the glutathione/ glutathione disulfide ratio (GSH/GSSG), and measurement of ROS using 2′,7′-diclorodihydrofluorescein diacetate. Other assays such as8-hydroxy-2' -deoxyguanosine (8-OHdG) detection via HPLC-electrochemical detection are used to assess oxidative DNA damage. Lipid peroxidation, another indicator of oxidative stress, can be measured by thiobarbituric acid-reactive substances (TBARS assay) for detection of malondialdehyde (MDA), and detection of modified lipids by matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS). Many agents and chemicals are capable of inducing oxidative stress.

Examples include estrogen-only menopausal therapy; ciclosporin; biological agents such as the Epstein - Barr virus and schistosoma haematobium; tobacco smoking; and dioxin.

3. **Protein adducts.** Protein adducts are complexes formed when chemicals covalently bind to protein molecules mainly through an electrophilic attack of a xenobiotic on the nucleophilic centers of proteins. Protein adducts are biomarkers of exposure to active xenobiotics, which can also produce DNA adducts and lead to mutations (Meyer and Bechtold 1996). They are sometimes considered as indirect indicators of DNA damage. Alterations in protein function caused by adducts can also disrupt cellular control, which can lead to cancer. The immunocomplex enzyme (ICE) assay is used for the detection of DNA–protein covalent complexes (DPCCs). Examples of agents that induce protein adducts include cigarette smoke and aflatoxin.
4. **Clastogenic Effects.** Clastogenic effects involve breaks in chromosomal material, or the rearrangement, gain or loss of pieces of chromosomes (Snyder 2010). Different forms of clastogenic effects include in vivo or in vitro chromosomal aberrations, micronuclei formation, aneuploidy, and abnormal karyotypes. Most of these biomarkers can be examined microscopically in different assays, both in vivo and in vitro. Chemotherapeutic drugs (such as chlorambucil) and certain occupational exposures such as to 4-aminobiphenyl, benzidine, 2-napthylamine, or formaldehyde, provide examples of agents that induce clastogenic effects.
5. **Gene Mutations.** Gene mutations refer to changes in the normal nucleotide sequence of DNA within a cell. Gene mutations play a central role in human cancers (Ding et al. 2008; Vogelstein and Kinzler 2004). Mutations can be silent or produce alterations in mRNA leading to abnormal protein expression. They are usually caused by copying errors during DNA replication (often due to the presence of DNA adducts) or as result of DNA damage such as strand breaks that could not be repaired by DNA repair mechanisms. These mechanisms often lead to base substitution, insertion, or deletion of one or more base pairs. They can produce major chromosomal restructuring (see endpoint #4: clasotgenic effects). Mutations related to carcinogenic mechanisms can occur in oncogenes (e.g. k-ras), tumor-suppressor genes (p53, Tsc, VHL) or genomic instability genes (e.g. DNA repair genes). Many toxicological tests are used to detect mutations, including in vitro assays such as Ames assay , and in vivo tests such as transgenic rodent assays (MutaMouse, BigBlue rat or mouse), and the somatic mutation and recombination test [SMART] assay which identifies somatic mutations in wing cells (wing-spot test) and eye cells (eye-mosaic assay system). Many agents are capable of inducing gene mutations, including chemotherapeutic agents, heavy metals (beryllium), most of radioactive agents, tobacco smoke, aflatoxins, and a variety of occupational agents (including arylamines and benzo[a]pyrene).
6. **Epigenetic alteration**s. Epigenetic alterations are stable, long-term alterations in the transcriptional potential of a cell that results in changes gene expression or chromatin structure, without changes in DNA sequence. Epigenetic mechanisms are involved in many normal cell processes, including embryogenic development and cell differentiation. Epigenetic alterations are associated with the development of many diseases, including cancer (Hamm and Costa 2015). These epigenetic effects can be manifested as altered methylation of DNA, changes in miRNA expression, and changes in chromatin and histone structure. Their detection normally involves examining changes in expression of DNA, DNA methylation status, and detection of mutations in proteins/enzymes controlling this

apparatus. Diethylstilbestrol, hepatitis B and C viruses, asbestos, some types of radiation, and dioxin provide examples of agents that induce epigenetic effects.

7. **Changes in gene expression**. This endpoint refers to alterations in the levels of expression of genes that are active in the cell cycle and related facets of cellular function. These changes frequently arise through from epigenetic effects (Garnis et al. 2004). They can also arise from a direct effect of the agent or through alterations in intracellular signalling. Alterations in mRNA or miRNA expression in relevant genes or pathways or epigenetic changes in genomic instability genes (DNA replication and repair genes) can be detected by several assays involving quantification of mRNAs or expressed proteins. Many agents are capable of inducing changes in gene expression, including estrogen therapies, viral agents, heavy metals (arsenic), cigarette smoke, and diesel engine exhaust,
8. **Alterations in cell signaling pathways**. This endpoint relates to the ability of an agent to interfere with cell signalling pathways, leading to expression of a carcinogenic phenotype in the cell. Altered cell signalling pathways can lead to evasion of mechanisms that limit cell proliferation (e.g. apoptosis and replicative senescence) and may ultimately result in the facilitation of cell invasion (Martin 2003). Some alterations in signalling pathways are central to cancer development (Bianco et al. 2006), including: the ras pathway, the COX-2 pathway, the mitogen-activated protein kinase (MAPK) pathway, and ATM-p53. A wide variety of assays and tests are used to measure alterations in cell signalling. The majority of those are based on measurements of the concentration of protein and non-protein second messengers (Ras proteins, cAMP, and calcium). Examples of agents that induce this type of effect include estrogen therapies, heavy metals (arsenic and beryllium), biological agents (Epstein Barr virus, Hepatitis C virus) and dioxin.
9. **Metabolic activation**. This endpoint applies when metabolic activation of the agent through the formation of electrophiles is necessary for carcinogenesis. The agent itself is not itself reactive with DNA or other key cellular components. Instead, it requires biotransformation (metabolic activation) by enzymes in organs such as the liver to produce metabolites that are active carcinogens (Miller 1970). Examples of metabolic activation include: formation of an alkylating agent, oxidation to epoxide metabolites, and formation of arylnitrenium ions. This endpoint can often be identified through metabolism studies of the formation and elimination of electrophilic metabolites, and is often indicated by a positive result in mutagenesis assays that only occurs in the presence of liver extract and/or in the presence/absence of glutathione. Benzo[a]pyrene is an example of an agent that requires metabolic activation to induce genotoxic through the formation of an electrophilic metabolite.
10. **Susceptibility**. Susceptibility refers to individual variation in the risk of developing cancer. Susceptibility can arise from a range of factors including: the presence of one or more inherited gene mutations (often marked by a family history that indicates an increased risk of disease) or exposures early in life (i.e. transplacental, in utero, early postnatal or lactational exposure) (Anderson et al 2000). Genotype susceptibility can be measured in vivo by SNPs, and detection of genetic polymorphisms in critical genes.
11. **Immune effects.** The immune system is a key factor in the response of the body to exposure to exogenous agents, particularly viral, bacterial and parasitic organisms. Adverse effects on the functioning of the immune system can result from exposure to

infectious agents such as HIV as well as to chemical substances. Altered immune function, such as that associated with ageing (Fulop et al. 2010), may lead to the increased incidence or severity of infectious diseases or cancer, since the immune system's ability to respond adequately to invading agents is suppressed. The immune system also plays a major role in the inflammatory response to injury: such inflammatory response can release cytokines and other factors that contribute to carcinogenesis. Carcinogenic agents that perturb the immune system include combined estrogen-progestogen menopausal therapy and dioxin.

12. **Chronic inflammation**. Many cancers arise in sites of chronic inflammation, which provide a long-term stimulus to the immune system. As noted previously, the response of the immune system to injury releases factors that contribute to carcinogenesis (see endpoint #11: immune effects). Chronic inflammation can lead to oxidative DNA damage. The tumour microenvironment, which is largely orchestrated by inflammatory cells, is an indispensable participant in the neoplastic process, fostering proliferation, survival and migration (Coussens and Werb, 2002). In addition, tumour cells have adopted some of the same signalling molecules of the innate immune system, such as selectins, chemokines and their receptors to cause invasion, migration and metastasis. Assays that evaluate this endpoint include: light microscopy, cytokine assays, and gene-ex13. Agents that induce cancer through inflammatory pathways include hepatitis viruses, the Epstein-Barr virus, and Schistosoma haematobium.
13. **Cell death**. Programmed cell death (apoptosis) is one of mechanisms by which a cell protects itself from DNA damage. Defects in programmed cell death can cause cancer (Kelly and Strasser 2011). In the presence of severe damage, the cell initiates a cascade of events that leads to the destruction of the cell. The tumor suppressor gene p53 plays a major role in the integrity of this process. Evasion of apoptosis is a requirement for both neoplastic transformation and sustained growth of cancer cells (Hanahan and Weinberg, 2000; Weinberg, 2007). Alterations in apoptosis (including both inhibition and induction of apoptosis, autophagy, and necrosis) can be identified by a large number of assays. These include apoptosis-specific assays, such as: TUNEL (TdT-mediated dUTP Nick-End Labeling) assay, DNA-ladder analysis for detection of DNA fragmentation, detection of apoptosis-related proteins (p53, Fas, Bcl-2/Bax ratio, cytochrome c, caspases), and cytotoxicity and cell viability assays such as clonogenic cell survival, ATP-based bioluminescence assays, and light-microscopic evidence of necrotic nuclei. Examples of agents that either induce or interfere with cell death include aristolochic acid; viral agents such as the Epstein-Barr virus, hepatitis viruses, and herpes virus; and arsenic, asbestos, and benzidine.
14. **Chronic irritation.** Chronic irritation can arise from a variety of external factors such as repetitive trauma and exposure to acid (e.g. gastric acids). These factors create an environment of chronic inflammation which contributes to cancer (see end point #12: chronic inflammation). Chronic irritation is strongly associated with certain types of gastrointestinal tumors such as forestomach (stomach/esophageal) cancer (Proctor et al. 2007). Agents that induce irritation include Oposthorchis viverrini, Schistosoma haematobium, asbestos and wood dust.
15. **Cell-cycle effects**. Cellular replication is controlled by a complex network of agents that regulate the cell cycle of division. These agents are responsible for preventing cell division in the presence of unrepaired DNA damage. Cell-cycle effects in cancer causation

refers to an alteration of the functioning of this complex series of signalling pathways, which has been associated with carcinogenesis (Diaz-Moralli et al. 2013). Detection of alterations in cell proliferation and cell-cycle effects (e.g., DNA replication changes, cell-cycle control) can be achieved by replicative DNA synthesis (RDS), BrdU labeling, proliferating cell nuclear antigen (PCNA) labeling, and flow cytometry. Agents that induce cell-cycle effects include arsenic and inorganic arsenic compounds, polycyclic aromatic hydrocarbons (PAHs), and Helicobacter pylori.

16. **DNA-repair alteration.** Cells are endowed with multiple mechanisms to preserve genome integrity. These can involve repairing DNA, which can be damaged by the formation of adducts, strand breaks, or other abnormalities. Key DNA repair mechanisms include base excision repair (BER) and nucleotide excision repair (NER). Inherited abnormalities in DNA repair function lead to enhanced cancer susceptibility. Recent preclinical studies provided evidence that multiple conventional DNA repair pathways are frequently altered in cancer (Dietlein et al. 2014). A variety of assays can be used to assess alterations in DNA repair mechanisms including inhibition of DNA-repair enzyme production or activity, loss of fidelity, and mutations in DNA repair enzymes. A number of agents interfere with DNA repair mechanisms, including Hepatitis C virus, arsenic, and beryllium.
17. **Receptor-mediated effects.** Receptor mediated effects are those that occur when an extracellular signalling molecule activates a specific receptor located on the cell surface or inside the cell. In turn, this receptor triggers a biochemical chain of events inside the cell, creating a response. Depending on the cell, the response alters the cell's metabolism, shape, gene expression, or ability to divide. Receptor-mediated effects are central in estrogen-induced breast cancer (Santen et al. 2014), and in the biological action of dioxin. Assays for agonists binding to receptors (e.g the Ah receptor, estrogen receptor, and androgen receptor) and activation of the downstream signaling pathways and/or induction of the biological effect of the estrogens and androgens are employed to reveal the ability of agents to induce receptor effects. Agents that induce receptor-mediated effects include hormonal therapies and dioxin.
18. **Hormonal Effects:** Hormones are chemicals, secreted by the body, that control the homeostasis, reproduction, development, and/or behavior of local or remote tissues (e.g. insulin and the control of glucose metabolism). External agents can interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body. Such agents can also demonstrate reactivity similar to endogenously produced hormones, which can lead to changes in homeostasis, reproduction, development, and/or behaviour. Hormonal therapies such as oral contraceptives and dioxin are examples of agents inducing hormonal effects.
19. **Angiogenic effects.** Angiogenesis refers to the process of inducing blood vessel growth. This is a normal physiological function that is essential for growth and maintenance of organs and body tissue. Tumor growth requires the induction of new blood vessel growth in the tumor through the secretion of various growth factors (e.g. VEGF) (Carmeliet et al. 2011; Saharinen et al. 2011). (This is commonly considered to be a hallmark of a tumor rather than a characteristic effect of an exogenous agent) Neovascularization of tumor tissues in treated animals is an assay commonly used to evaluate angiogenic effects. Agents that induce such effects include HTLV-1, nicotine, and NNK.

20. **Alterations in telomere length**. Telomeres occur at the end of human chromosomes and consist of repetitive DNA sequences that facilitate replication of the ends of chromosomes. However, during each cycle of DNA replication, the length of the telomere is reduced. Eventually, the reduction in length leads to cellular senescence. Carcinogenesis involves activation of a telomerase that prevents loss of telomere length, leading to immortalization of cells (Willeit et al. 2010). Reverse transcriptase-polymerase chain reaction analysis of extracted RNA is used to measure expression levels of the telomerase components. Ionizing radiation has been shown to induce telomere stabilization.
21. **Inhibition of gap-junctional intercellular communication**. Gap junctions are plaque-like features on the cell plasma membrane. Complexes of adjacent cells can physically combine, providing a channel through which electronic signals and various signalling molecules (e.g. ions, second messengers, and low molecular weight metabolites) can pass from the interior of one cell to the other. This facilitates coordination of cellular metabolism and maintenance of homeostasis. Disruption of these communication pathways can cause a loss of 'contact inhibition' and abnormal cell growth (Loewenstein et al. 1996). Indicators of gap-junction effects include oncogenic transformation (i.e., anchorage-independent growth, and loss of contact inhibition). Assays and test system typically used to assess this endpoint include Syrian hamster embryo cells, cultured mouse fibroblast cells, Gap-junctional intercellular communication (GJIC): inhibition of metabolic cooperation or of dye transfer, increased motility and invasiveness of cancer cell lines. Helicobactor pylori, PAHs, and HTLV-1are known to interfere with gap-junctional intercellular communication.
22. **Bystander effects.** The bystander effect was first identified in radiobiology and refers to the situation where non-irradiated cells in close proximity to irradiated cells exhibit the effects caused by radiation as a result of chemical signals (messengers) received from nearby irradiated cells (Prise and O'Sullivan 2009). These effects are often mediated through gap-junction transfer of chemical agents. Radiation is the best example of agents that induce bystander effects.
23. **Immortalization.** Immortalization refers to a cellular stage in which the cell can evade normal cellular senescence and will proliferate indefinitely. It is frequently associated with activation of telomerase (Willeit et al. 2010), and plays a critical role in carcinogenesis (Reddel, 2000). This effect is usually manifested as oncogenic transformation (i.e., anchorage-independent growth, loss of contact inhibition). Immortalized hepatocytes from transgenic mice are widely used to study various toxicological responses including carcinogenesis (Amicone et al. 1997; Sacco et al. 2004). Agents that induce immortalization include HIV and HPV.
24. **Absorption, distribution, metabolism and clearance differences.** Absorption, distribution, metabolism and elimination (ADME) of an agent can affect the bioavailability of the active carcinogen at the site of action, and therefore impact its carcinogenicity. Pharmacokinetics/toxicokinetics (PK/TK), mass balance studies, quantitative tissue distribution studies, metabolic profiling and identification are methods to assess the effects of metabolism on the activation of carcinogens. Toxicokinetic factors modify the carcinogenic potential of many Group-1 agents.

Perspectives on the Toxicological Endpoints

Accumulating evidence from molecular epidemiology studies shows that the risk of chemically induced cancers varies in different individuals as a function of inherited factors (i.e. individual genotype) as well as acquired factors (e.g. environmental exposures) (Rothman et al. 2001). Risk is also affected by other factors specific to the agent such as exposure conditions (dose, frequency and duration of exposure) and the host's health and nutritional status. This variation in risk is most likely mediated by the key mechanistic pathways involved in carcinogenesis.

Cancer risk may be different in different life stages. Exposures to external agents can elicit different types and levels of toxicological endpoints according to the age of the exposed individuals. This may be related to different levels of exposure: the risk of ovarian cancer, for example, varies in response to reproductive characteristics such as hormonal use and menopausal status (Moorman et al. 2008). In other cases, the risk of cancer may change as a result of age-related changes in cellular structure or function. For example, the functioning of the chromatin apparatus appears to change during the ageing process (Das and Tylor 2013), which would lead to changes in genomic functions such as transcription, replication and repair. DNA repair is another molecular process that decreases in effectiveness with age (Garm et al. 2013).

Gender is also an important factor that affects response to carcinogen exposure. Hochstenbach et al. (2012) reported gender differences in response to carcinogen exposure *in utero*. In that study, gender-specific differences were observed in gene expression associated with dietary genotoxic and non-genotoxic exposures linked with the cell cycle, the immune system, and more general cellular processes such as post-translation. Levels of DNA methylation induced by prenatal cadmium exposure have been shown to differ between males and females (Kippler et al. 2013). Gender differences in DNA repair has also been reported in mice and rats exposed to 1,3-butadiene (Swenberg et. al. 2011).

Genotype differs by race/ethnicity, and, consequently, the prevalence of variants in genes of critical metabolic enzymes or signaling molecules is different in different population groups. This may contribute to explaining variations in incidence of cancers in different populations exposed to similar types and levels of carcinogenic agents (Park et al. 2014; 2015; Derby et. al. 2009).

Gene-environment interactions are also apparent in biomarkers of biological effects such as DNA adducts (Iyer et al. 2014; Nock et al. 2007). Genetic variants can also interact with other molecular mediators of pathways related to apoptosis, cell proliferation and neoplastic processes, these being key mediators in heavy metal mutagenicity and carcinogenicity (Kwon et al. 2013; Koedrith et al. 2013). Polymorphisms in key enzymes involved in metabolism of carcinogenic agents (such as cigarette smoke) may play a role in susceptibility to a number of cancer sites: examples include polymorphisms in cytochrome P450 and arylamine N-acetyltransferase (NAT) in head and neck cancers (Khlifi et al. 2013), CYP1A1 in squamous lung carcinoma (Ji et al. 2012), and CYP1A1 in cervical cancer (Roszak et al. 2013). Furthermore, low-level environmental exposures may be more relevant in genetically susceptible individuals. For example, polymorphisms of the CYP1A1 and glutathione S-transferase genes conferred increased risk of lung cancer in relation to lower levels of

cigarette smoking (Nakachi et al. 1993). The slow acetylator phenotype was associated with decreased clearance of the bladder carcinogen 4-aminobiphenyl after low dose exposure (Vineis et al. 1994). This has importance for risk assessment where differences in levels and types of biomarkers and hence cancer risk may be related to both environmental (e.g. dose) and host (e.g. genotype) factors.

Human exposure to environmental agents commonly involves exposure to complex mixtures of a diverse range of chemicals (e.g. cigarette smoking and certain industrial manufacturing processes). Of these exposure mixtures, some are carcinogenic (e.g. air pollutants, diesel engine exhaust, wood dust, cigarette smoke). Cigarette smoke contains more than 7,000 different chemicals. Painting involves exposure to over a thousand different substances. As the composition of the environmental mixtures can vary to a great extent in terms of chemical structure and physical characteristics (e.g. particle size distribution, gaseous and particulate components), their biological activity can also vary. Furthermore, the composition of many of these mixtures varies across industrial sites and sectors and with the changing nature of many industrial processes. The presence of different chemicals in these mixtures implies that the toxicological endpoints involved would be more diverse than those associated with exposure to the separate constituents of the mixture. Furthermore, chemical interactions between chemicals in complex exposures are possible, as outlined below. The evidence relating to the mechanistic pathways involved in carcinogenicity of specific chemical compounds (e.g. benzo[a]pyrene) in mixtures was rarely obtained from human exposures; rather, epidemiological evidence tends to be available only for mixtures containing the specific compound of interest. This evidence, along with data on structure activity relationships and experiments on animals, is used to deduce the mechanism(s) of action of the agent.

Many examples of interaction among multiple exposures exist in the literature on co-exposures and resulting biological responses. Arsenic was found to potentiate benzo[a]pyrene genotoxicity by inducing DNA adducts in mouse hepatoma Heap-1 cells (Maier et al. 2002), and by enhancing oxidative stress in human lung adenocarcinoma cells (Chen et al. 2013). Interaction can occur between radiation and heavy metals as measured by DNA damage in human keratinocytes and in SKH-1 hairless mice (Cooper et al. 2013). Another example is the synergistic effect of alcohol and tobacco smoke associated with an increased risk of upper digestive tract (Pelucchi et al. 2006). More complex interactions involving biological agents can also occur. Examples include the interaction between aflatoxin exposure and chronic hepatitis B viral (HBV) infection which increases the risk of hepatocellular carcinoma via a variety of potential mechanisms including increased frequency of p53 mutations (Kew 2003), and increased levels of DNA adducts (Chen et al. 2001). Similarly, environmental PAH exposure appear to increase the risk of hepatocarcinogenicity among those with high aflatoxin exposure and chronic HBV infection (Wu et al. 2007). There is also evidence that Chinese-style salted fish can re-activate the Epstein Barr virus, which is known to cause nasopharyngeal carcinoma.

The level of exposure plays an important role in carcinogenesis. Whereas experiments with high toxic doses of inorganic arsenic show little evidence of a mutagenic response, long term, low-dose exposure to inorganic arsenic may cause increased mutagenesis, perhaps mediated by increased levels of reactive oxygen species, as well as co-mutagenesis with other agents.

Other underlying mechanisms observed at low concentrations of arsenic include DNA-repair inhibition, and slower changes in DNA-methylation patterns, aneuploidy, and gene amplification. Clastogenic effects have been observed after low dose exposure to beryllium, but not at higher doses. In contrast to the low-dose effects in the previous two examples, nickel induced-genotoxic effects (e.g. sister chromatid exchange, chromosomal aberrations, and micronuclei) have been observed only at toxic levels.

The role of dietary habits and nutritional status (e.g. dietary intake of fruits and vegetables) and antioxidants levels on chemical carcinogenesis has been investigated in a number of studies. Some have shown a protective effect, owing mainly to a reduction of oxidative DNA damage (Loft et al. 2008). The level of intake of dietary antioxidants was reported to modulate the association between polycyclic aromatic hydrocarbons and DNA adducts. This modulation differed by allele variants in the DNA base excision repair gene (Shen et al. 2005). Individual anthropometric measures (e.g. body mass index) affect the levels of circulating insulin and insulin-like growth factors, thus potentially modulating background cancer risk (Moore et al. 2012).

Key Characteristics of Carcinogenic Agents

Description of the Ten Key Characteristics

In this section, we describe the 10 key characteristics of carcinogenesis developed by the Working Group at its second meeting, and subsequently elaborated by Smith et al. (2015). This list is not meant to be exhaustive, but was recognized by the Working Group as representing important characteristics of human cancer. Nor are the key characteristics mutually exclusive: as demonstrated by Krewski et al (2015), this volume), many of the Group-1 agents demonstrate multiple characteristics. , The10 key characteristics of human cancer articulated by Smith et al. (2015) are listed in Table 2, and described below.

[insert Table 2 about here]

1. **Electrophilicity and Metabolic Activation.** Electrophilicity is a chemical characteristic of many direct acting carcinogenic agents; for others, it is a characteristic of their metabolites (Miller et al. 1970). The reaction between an electrophile and a target macromolecule such as DNA usually results in the formation of adducts. Examples of direct acting electrophilic carcinogens include formaldehyde, sulfur mustards, and ethylene oxide. Agents that become electrophilic after biotransformation include many polycyclic aromatic hydrocarbons (Hecht, 2012; O'Brien, 2000).
2. **Genotoxicity.** Genotoxic agents are able to induce DNA damage that leads to the formation of DNA adducts, as well as single or double strand breaks. These DNA lesions may or may not develop into mutations, depending in part on the capacity for and efficiency of for DNA repair. Most of the Group 1 human agents are considered to be genotoxic and many are mutagenic (Waters et al., 2010). All electrophiles are genotoxins, but not all genotoxins are electrophiles.

3. **Altered DNA Repair and Genomic Instability**. Defects in processes that determine DNA replication fidelity can confer a strong mutator phenotype that results in genomic instability. Carcinogens may act not only by producing DNA damage directly, but also by altering the processes that control normal DNA replication. Normal cells avoid deleterious mutations by replicating their genomes with high accuracy. DNA is repaired by a number of mechanisms including base excision repair, nucleotide excision repair, and double strand break repair. Failure to repair DNA damage results in genomic instability, which is manifest as chromosome aberrations, gene-sequence and mini-satellite mutations, and apoptosis. Genomic instability is a well-recognized feature of many cancers (Bielas et al., 2006). An example of agent that impairs DNA damage repair resulting in genomic instability is ionizing radiation.
4. **Epigenetic Alterations.** Cellular epigenetic mechanisms represent another tier of control of gene expression. However, unlike control of gene expression conferred by gene regulation sequences on DNA, epigenetic control functions independently of the DNA sequence and involves multiple levels of regulation. These include genomic imprinting, X-chromosome inactivation and global reconfiguration of the DNA methylome, changes in chromatin compaction states, and histone modification patterns. These mechanisms can be inherited through cell division and are maintained over the lifetime of an organism. Many of these same phenomena have been shown to be altered during carcinogenesis. A wide range of known and suspected carcinogens (including chemical, physical and biological agents) have been shown to deregulate the epigenome. It has been suggested that their mode of action may involve disruption of epigenetic mechanisms. Examples of Group 1 agents that induce epigenetic changes during the carcinogenic process include diethylstilbestrol, hepatitis B virus, human papilloma virus, nickel, fibers, radiation, ethanol and benzo[a]pyrene. However, evidence for a truly causal role of epigenetic changes in cancer produced by Group 1 agents is currently limited (IARC, 2012abcdef). For many agents, their impact on the epigenome was considered to be a secondary mechanism of carcinogenesis; however, this might be because these mechanisms have not been investigated until recently.
5. **Oxidative Stress.** Oxidative stress can be defined as an imbalance in reactive oxygen formation and detoxification. The resulting reactive oxygen species induce a cascade of events that can include DNA mutation and oxidative DNA damage. Both are key events in carcinogenesis (Klaunig et al., 2011). Oxidative damage to DNA can play a critical role in carcinogenesis by formation of point mutations, deletions, insertions, or chromosomal translocations, which may cause oncogene activation and tumor suppressor gene inactivation (Klaunig et al., 2011, Berquist and Wilson, 2012).
6. **Chronic Inflammation:** Chromic inflammation alters cell signaling and leads to the recruitment of inflammatory cells. These ultimately release agents that induce oxidative stress and genomic instability. This may be the pathway linking chronic inflammation to cancer (Multhoff and Radons, 2012). In some instances, inflammation becomes a secondary event driven by activation of protooncogenes in preneoplastic and neoplastic cells, which then recruit host-derived inflammatory cells that accelerate tumor promotion and progression (Grivennikov et al., 2010). Inflammation is one of the hallmarks of cancer (Hanahan and Weinberg, 2011) and it has been hypothesized to contribute to both the initiation, promotion and progression of cancer (Trinchieri, 2012).

Examples of carcinogenic agents that induce chronic inflammation include Helicobacter pylori, different viral infections, silica, and asbestos fibers.

7. **Immunosuppression.** Several Group 1 agents (e.g. human immunodeficiency virus (HIV-1) and the immunosuppressive drugs cyclosporin and azathioprine) suppress immune system functioning. Immunosuppression compromises the capacity of the immune system to respond effectively to foreign antigens, including antigens on tumour cells. It also renders the host susceptible to infection by agents which are carcinogenic (e.g. Kaposi's sarcoma virus).
8. **Receptor-Mediated Effects.** Agents that mimic the structure of endogenous ligands can bind and activate cell surface or intracellular receptors and induce or modify a wide range of cell transduction pathways that increase cell proliferation. Some genotoxic compounds are also able to bind to cell or nuclear receptors. The best examples are polycyclic aromatic hydrocarbons (e.g. benzo[a]pyrene) which bind to the aryl hydrocarbon (Ah) receptor. The Ah receptor is an example of a receptor with no known endogenous ligand that is activated by many xenobiotics. Receptor mediated effects may also involve indirect pathways (e.g. modulation of the amount of endogenous ligand available for binding) and the activation of a receptor affecting biosynthesis, bioavailability, bioactivation, and degradation of the bioactive ligand.
9. **Immortalization**. In cell culture, normal cells have a fixed number of replication cycles before they enter cellular senescence and stop replicating. Immortalization refers to a stage where the cell can evade normal cellular senescence and will proliferate indefinitely. It is frequently associated with activation of telomerase (Willeit et al. 2010), and plays a critical role in carcinogenesis (Reddel, 2000). This effect is usually manifested as oncogenic transformation (e.g. anchorage-independent growth and loss of contact inhibition). Immortalized hepatocytes from transgenic mice are widely used to study various toxicological responses including carcinogenesis (Amicone et al. 1997; Sacco et al. 2004). Several human DNA and RNA viruses that are carcinogenic to humans induce immortalization, including various human papillomaviruses, Epstein-Barr virus, Kaposi's sarcoma-associated herpesvirus, hepatitis B virus, hepatitis C virus, and human immunodeficiency virus (IARC, 2012b). These viruses have evolved multiple molecular mechanisms that disrupt specific cellular pathways with resultant aberrant replication. Although oncogenic viruses belong to different families, their involvement in human cancer development show many similarities and include viral-encoded oncoproteins that target key cellular proteins that regulate cell growth.
10. **Altered Cell Proliferation or Death.** Some carcinogenic agents induce altered cellular proliferation: this may be either a direct effect on cell proliferation or a secondary (regenerative or compensatory) effect after induction of cell death by cytotoxicants. Increased cellular proliferation may confer a growth advantage to mutated cells. An increased rate of mutations may also occur subsequent to rapid cellular proliferation. It has been reported that mutagens are more potent carcinogens when administered at doses that also induce cell proliferation (Butterworth et al. 1992). Cell death can occur through a number of pathways, including apoptosis, autophagy, and necrosis. The first two of these three pathways are regulated, while necrosis usually occurs subsequent to cell injury. Apoptosis or its resistance plays a vital role in carcinogenesis. There are many ways a malignant cell can acquire reduction in apoptosis or apoptosis resistance. One of these pathways involves disrupted balance of pro-apoptotic and anti-apoptotic proteins

(Wong, 2011). For example, aberrant function, mainly due to mutations, of p53, which is a key regulator of apoptosis, contributes to marked proliferation of cells (Slater et al. 2011). Necrosis is associated with release of pro-inflammatory signals into the surrounding tissue microenvironment resulting in recruitment of inflammatory cells of the immune system. These cells, in turn, release cytokines and other inflammatory mediators that participate in tumor promotion by induction of cancer cell proliferation and invasiveness.. Examples of agents that induce cell proliferation during carcinogenesis include Tamoxifen, diethylstilbestrol, estrogen therapy, a number of viral agents, beryllium, fibers, benzidine, and dioxin.

Perspectives on the Ten Key Characteristics

Some of the key characteristics of carcinogens described above can be considered themselves as primary events that trigger the carcinogenic conversion, including agents facilitate the original mutagenic changes in stem and progenitor cells that initiate the cancer process. These are distinct from those enhancing the relative rate of growth vs differentiation/death of initiated clones (e.g. inhibition of growth-suppressing cell-cell communication) in terms of their carcinogenic potency and subsequent relevance to cancer as an endpoint (Chen et al 2014). There are other key characteristics that may occur at later stages of chemical carcinogenesis (e.g. enhancing the growth, malignancy, or spread of already developed tumors through suppression of immune surveillance; hormonally mediated growth stimulation for tumors with appropriate receptors by estrogens). Epigenetic silencing of tumor suppressor genes may occur as multiphasic during the various steps in the carcinogenic process (Hattis et al. 2009). These distinctions have important implications for cancer risk assessment, since agents which exhibit characteristics that induce early changes in the carcinogenic process may be more strongly associated with cancer as an endpoint than those that those characteristics effecting later stages on cancer conversion.

Similarly, some of the mechanistic characteristics of carcinogenic agents may correlate better with cancer risk than others. Agents which react directly with DNA may form DNA adducts or induce single or double DNA strand breaks; such genotoxic effects common to most of Group 1 carcinogens. Several lines of evidence from epidemiological studies, experimental animals, and model systems have shown that DNA adducts are strongly associated with cancer (Wiencke et al. 2002; Kriek et al. 1998, Phillips et al. 2014). Genotoxic effects can also lead to mutations. As noted above, gene mutation represents an important event in the pathway of carcinogenesis, especially if it involves oncogenes or tumor suppressor genes. Ras mutations that result from exposure to polycyclic aromatic hydrocarbons are involved in the etiology of tumors (Ross and Mesnow 1999), and mutations in p53 from other chemical exposures are linked to human cancers (Hussain and Harris 1999). Chromosomal changes are another type of genetic alteration frequently seen in many tumors. Consequently, agents that induce genomic instability (e.g. benzene) should be regarded as potential carcinogens.

Other characteristics of carcinogenic agents (e.g. changes in gene expression, altered cell proliferation, immunosuppression, inflammation and oxidative stress) may not reliably

predict carcinogenicity (Melnick et al. 1996, Hernández et al. 2009). One reason could be that those events have also been implicated in relation to other adverse health outcomes, and therefore lack specificity with respect to cancer. Alternatively, these agents may be active during the process of neoplastic conversion and therefore they could, in some cases, be associated with the carcinogenic conversion process rather than acting as initiating events (Hanahan and Weinberg 2011; Amend and Pienta, 2015; Brücher and Jamall 2014). A number of factors such as dose and exposure frequency and duration may influence whether and what toxicological endpoints would be elicited by specific agents and determine their relative contribution to the overall process of carcinogenesis (Bolt and Huici-Montagud, 2008).

It is worth noting that there may be exceptions to the notions outlined above regarding relevance of the different key characteristics of carcinogens. For example, Labib et al. (2012) reported that early changes in gene expression may provide a better indication of the likelihood of carcinogenic transformation than DNA adducts and mutant frequency in response to benzo[a]pyrene exposure. Nonetheless, the 10 key characteristics were considered by the WG to provide useful descriptors of the properties of Group-1 agents that are related to the biological mechanisms by which such agents cause cancer in humans.

Linking the Toxicological Endpoint to the Key Characteristics

In constructing the IARC database of mechanisms of human cancer, data on the 24 toxicological endpoints relevant to carcinogenesis were abstracted from the IARC Monographs, with supplementary information gathered through a PubMed search. In order to construct a database on the 10 key characteristics developed by Smith et al. (2015), the 24 toxicological endpoints were linked to the 10 key characteristics, as shown in Table 3. For example, toxicological endpoint #6, which includes epigenetic changes such as altered methylation of DNA, miRNA expression, and changes in chromatin and histone structure was linked with key characteristic #4, epigenetic alterations. As seen in Table 3, more than one toxicological endpoint may link to a single key characteristics, reflecting the broader scope of the key characteristics than the toxicological endpoints in describing factors related to the development of cancer in humans. Two toxicological endpoints susceptibly (toxicological endpoint #10) and changes in gene expression (toxicological endpoint #7) were not considered to be specific to any of the 10 key characteristics, and were thus not included in the linkage between the toxicological endpoints and the key characteristics. The database on the key characteristics of human cancer constructed in this manner was used by Krewski et al. (2015) to explore the characteristics of 86 Group-1 agents identified in the IARC Monographs through Volume 107, the last volume for which the final version of the Monograph was available at the time the mechanistic database was constructed.

[insert Table 3 about here]

Discussion

In reviewing the different toxicological endpoints elicited by Group 1 agents identified by the IARC and their key characteristics, distinct patterns are apparent for different groups of agents, with some agents exhibiting multiple and varied toxicological pathways and others demonstrating a more narrow range of pathways. . Based on these toxicological endpoints and key characteristics, agents can be broadly classified into either electrophiles or DNA reactive agents (often called genotoxic compounds) versus DNA nonreactive agents (often called non-genotoxic), depending on whether or not the endpoint involves interaction with the genetic material and subsequently causing DNA and/or chromosomal damage. Electrophiles and DNA reactive agents are generally chemically reactive. Electrophilic agents, or agents that are metabolized to electrophiles, are able to react chemically with the nucleophile-rich centers in the cell including nucleotides, generating DNA adducts.

In contrast, some agents cause DNA damage through indirect means. For example, they might influence the cellular redox processes, particularly nuclear redox state that works independently from the cytoplasmic one (Go and Jones 2010), producing reactive oxygen species that then react with DNA. Oxidative damage to DNA can lead to several lesions including mutations, single strand breaks, DNA-protein crosslinks, chromosomal abnormalities and translocations. Mutations via oxidative stress can activate oncogenes and deactivates tumor suppressor genes (Klaunig et al., 2011).

Examples of direct acting genotoxic agents include chemotherapeutic agents such as busulfan, chlorambucil, methyl-CCNU, melphalan, and the industrial agent ethylene oxide and Sulphur mustard. All of these are direct electrophilic alkylating agents. Examples of agents whose electrophilic metabolites reacts with DNA to form DNA adducts include benzo[a]pyrene (found in tobacco smoke, and some industrial processes), some therapeutic agents (e.g. Tamoxifen, Thiotepa, treosulfan), and some industrial agents and processes (4-aminobiphenyl, benzidine, dyes metabolized to benzidine, 2-naphthylamine, o-toluidine, auramine production, magenta production, coal gasification, coal-tar distillation, coal production). Heavy metals and fibers act mainly by inducing the formation of reactive oxygen species thus causing genomic instability, chromosomal aberrations, DNA strand breaks, DNA-protein cross-links.

Agents that are not chemically reactive (mainly lipophilic compounds) and biological agents (hepatitis viruses, human T-lymphotrophic virus, Opisthorchis viverrini , Schistosoma hematobium, and Helicobacter pylori) act via a variety of mechanisms, which generally involve interfering with cell signaling, resulting in varied molecular changes and end points such as altered cell proliferation and migration, disruption of apoptosis, changes in gene expression, cellular immortalization and transformation. Other non-genotoxic mechanisms involve interfering with the epigenetic apparatus by changing DNA methylation, or inducing histone modifications. Hormonal therapies and agents mimicking hormonal action (e.g. dioxin) induce receptor-mediated tissue specific, and agent specific cell proliferation, mitogenesis and other events.

The majority of Group 1 agents demonstrate genotoxicity as a key characteristic. The proportion of non-genotoxic carcinogens among known (Group 1), probable (Group 2) and possible (Group 3) human carcinogens classified by the International Agency for Research

on Cancer (IARC) was evaluated by Hernández et al. (2009): they found that 12% (45/371) of the agents Groups 1, 2A and 2B carcinogens had a non-genotoxic mode of action.

The classification of carcinogenic agents as genotoxic and non-genotoxic based on their mechanism of action is not always clear and distinct. Some agents interfere with multiple pathways that involve induction of DNA damage as well as other types of effects not involving DNA reactivity. Examples of the latter include changes in gene expression, activation of cell signaling pathways, immunosuppression, and inflammation. Furthermore, DNA damage from chemical exposures may be a secondary or tertiary effect of the cascade of events mediated through the agent's metabolism or its reaction with cellular constituents (e.g. receptor binding). For example, dioxin metabolism results in enhanced production of reactive intermediaries, which increase oxidative stress and subsequent oxidative DNA damage. Chronic inflammation from exposure to fibers results in genotoxic DNA damage. In another example, the chemotherapeutic agent etoposide produces genetic damage without chemically interacting with DNA. The postulated pathway in this case involves binding to the topoisomerase II enzyme. The etoposide-topoisomerase IIα complex interferes with DNA re-ligation, enhancing the production of DNA double-strand breaks. The same complexes can also directly block the advancing DNA replication fork resulting in sister chromatic exchange and aneuploidy. In some other cases, the prevalent non-genotoxic pathway may eventually lead to genotoxic event, as the case of HBV-related hepatocellular carcinoma (HCC) where increased cellular proliferation from inflammatory responses to chronic viral infection can produce double-strand DNA breaks and facilitate viral integration. Similarly, genomic instability events (such as chromosomal aberrations and micronuclei formation in response to heavy metals exposure, including arsenic, cadmium, beryllium and nickel), may result from interference with DNA repair.

For some agents, both a primary mechanistic pathway and a secondary pathway contributing to carcinogenesis can be identified based on chemical structure and in vitro experiments. However, the relative importance of each of these pathways to the carcinogenic process may be difficult to estimate in agent-induced human cancers. One reason is that multiple biomarkers and events are invoked during the carcinogenic conversion. An example of this is Tamoxifen, for which the evidence for the role of a genotoxic pathway in induction of human endometrial tumours is less compelling than the role of receptor-binding through an estrogen-receptor-dependent pathway. Similarly, for estrogen induced cancer, it is difficult to reach a conclusion as to whether it is the receptor-mediated responses to the hormone or the genotoxic effect of estrogenic hormones or their associated by-products

It becomes evident after reviewing the mechanisms of Group I agents included in Volume 100 of the IARC Monographs and beyond that multiple mechanisms operate for many carcinogenic agents. It is challenging to determine which predominates in the development of human cancers. Since mechanistic data has important implications for cancer risk assessment, including informing dose-response relationships, it is critical to consider the interrelationship of the key characteristics of human carcinogens, which may in turn also be informative with respect to the complex interactions among different carcinogens (Guyton et al. 2009).

Future Perspectives

Robust knowledge of the various mechanistic pathways and elicited toxicological endpoints during carcinogenesis is of vital importance in risk assessment of the carcinogenic agents. The focus in the next few decades will most likely be on the pathway(s) and molecular targets commonly modulated by exposure to carcinogenic agents that are relevant to the carcinogenic process. This approach will become more feasible with the application of molecular "omics" techniques to detect virtually all global changes in cellular constituents and processes after exposure to candidate carcinogenic agents. Furthermore, recent developments in molecular biology, especially in the area of high throughput molecular technology and advanced genome-wide scanning, hold the promise of fostering understanding of the major etiologic pathways and landmark events involved in cancer development. For example, recent genome-wide scan and analyses revealed the driver genes most commonly mutated in cancers: most of the signaling pathways they control and regulate have been identified (Vogelstein et al. 2013).

Understanding how pathways are perturbed, and determining which pathways are activated in different tumorigenesis settings, could have an important impact on understanding heterogeneity in response to carcinogenic agents. This may also lead to the development of more effective individualized tumor therapies and prevention strategies. Further, an interdisciplinary application of this research to molecular cancer epidemiology would help in the identification and subsequent validation of biomarkers along the pathway of chemical carcinogenesis, and could potentially identify common genetic variants determining inter-individual risk in cancer risk. Early detection in exposed individuals of mutations in genes controlling critical pathways may provide evidence of a "molecular abnormality" of potential carcinogenic conversion even before mutated cells undergo clonal expansion.

Well-validated biomarkers corroborated in multiple studies could be used for cancer prognosis, and reduction of cancer-related morbidities and mortality (Urlich et al. 2008). Genome wide association studies have been used to find genetic biomarkers associated with poor prognosis, as in colorectal cancer (Bacolod and Barany, 2011). For example, variation in cancer susceptibility is one of the toxicological endpoints considered in the current review (although it was not linked directly to any of the key characteristics of human cancer). It is an important biomarker of a range of host factors (mainly controlled by genotype variation) that affect the host response to carcinogenic agents. This endpoint potentially modulates all of the 10 key characteristics. It is an essential component of molecular epidemiological studies to understand inter-individual differences in susceptibility to chemical-induced cancer. Low-penetrant but high frequent gene allele variations controlling toxicokinetics (e.g. cytochrome p450 (Gold et al. 2009)), and to smaller extent toxicodynamics (e.g. estrogen receptor (Zheng et. al. 2003)), have an important impact on population attributable risk in chemical carcinogenesis. Variants in other genes contributing to cellular dysfunction (e.g. ras, p53 and BRCA1) have even stronger contributions to individual susceptibility to cancer. Knowledge on individual genetic variations paves the way for opportunities of more effective and individualized cancer therapy (Safgren et al. 2015).

A major challenge in the development and validation of reliable molecular biomarkers is to assess the background signals of genetic damage, especially that due to endogenously produced oxidative stress due to reactive intermediates of normal cellular metabolism

(Swenberg et al. 2011). The relative contribution of environmental and endogenous DNA damage to the overall carcinogenesis is difficult to determine but is important when attempted to reliably correlate exposure to exogenous carcinogenic agents with critical endpoints of carcinogenesis. However, major technological advances in high-throughput molecular technologies can be utilized to develop biobanks of integrated biological response (the concept of 'exposome') of people exposed to well-defined environmental agents, with the promise of earlier detection and eventual prevention of cancer (Wild et al. 2011). These techniques have been recently employed to understand the influence of environmental exposures on the epigenome (Marsit, 2015).

Tables

Table 1: Definitions of the 24 Toxicological Endpoints involved in Carcinogenesis and Prototypical Assays for each Endpoint.

Toxicological Endpoint	Definition	Description/Prototypical Assays
1. DNA damage	DNA damage is an alteration in the chemical structure of DNA, such as a break in a strand of DNA, a base missing from the backbone of DNA, or a chemically changed base such as 8-OHdG.	A. Direct Evidence of DNA damage—this category includes nuclear and mitochondrial DNA damage (*in vitro* or *in vivo*): 1. DNA adducts (NOTE: if detection is specifically for endogenously-produced oxidative adducts such as 8-oxodG, these will be classified under oxidative damage, see below) *Assays:* a. Detection of radiolabel in isolated DNA, typically ^{3}H or ^{14}C followed by liquid scintillation counting b. ^{32}P-postlabeling followed by high-performance liquid chromatography (HPLC) or thin-layer chromatography (TLC) c. Accelerator mass spectrometry d. Immunoassays e. Mass spectrometry (typically GC-MS) f. 2D liquid chromatography/tandem mass spectrometry (LC/MS/MS).

Toxicological Endpoint	Definition	Description/Prototypical Assays
		g. High-performance liquid chromatography (HPLC) interfaced with an electrochemical detector to detect depurinating adducts. 2. DNA strand-breaks (single- and/or double-strand breaks) *Assays:* a. Comet assay (single-cell gel electrophoresis) b. Alkaline elution assay c. Assays specific to DNA double-strand breaks, e.g., immunofluorescence of factors specific to dsbs (e.g., γH2AX) 3. DNA-protein cross-links; DNA-DNA cross-links a. Alkaline elution and comet assays b. Chromatin immuno-precipitation (ChIP) assays. c. Selective K sodium dedocyl sulphate (SDS)s precipitation of DNA associated with protein. B. Indirect indicators or biomarkers of DNA damage (*in vitro* or *in vivo*): 1. Sister chromatid exchange (SCE) 2. Unscheduled DNA synthesis (UDS)

Toxicological Endpoint	Definition	Description/Prototypical Assays
		3. Mitotic recombination and aneuploidy (mammalian and non-mammalian – e.g., *Saccharomyces cerevisiae*) 4. Chromosomal aberrations in plants (*Tradescantia, Allium, Vicia*) 5. Prokaryotic DNA damage and induction of DNA repair (e.g., umu test, prophage induction test, rec- differential survival test, SOS chromotest). 6. Formation of protein adducts [as indirect indicators of DNA adducts].
2. Oxidative stress	Oxidative stress is defined as a disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidant defenses within a cell. This compromises the cell's ability to detoxify the reactive intermediates or to repair the resulting damage. The effects of oxidative stress depend upon the size of these changes, with a cell being able to overcome small perturbations and regain its original state. However, more severe oxidative stress can cause cell death and even moderate oxidation can trigger apoptosis, while more intense stresses may cause necrosis. At a whole animal level, oxidative stress can be associated with a significant decrease in the effectiveness of antioxidant defenses, such as glutathione.	A. Cellular redox state *Assays:* 1. ROS measurement using DCFH-DA 2. Measurement of GSH/GSSG ratio 3. Measurement of reactive oxygen species (ROS) using 2′,7′-diclorodihydrofluorescein diacetate B. DNA oxidation, oxidative DNA damage *Assays:* 1. 8-oxodG detection via HPLC-electrochemical detection assay 2. Comet assay with formamido-pyrimidine DNA glycosylase (FPG) digestion C. Lipid peroxidation

Toxicological Endpoint	Definition	Description/Prototypical Assays
		Assays: 1. TBARS assay (thiobarbituric acid-reactive substances assay) for detection of malondialdehyde (MDA). 2. Detection of modified lipids by matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS). D. Oxidized proteins *Assays:* 1. Protein carbonyl colorimetric assay
3. Protein adducts	Protein adducts are complexes formed when chemicals bind to protein molecules. They are biomarkers of exposure to active xenobiotics which could also produce DNA adducts and lead to mutations. They are sometimes considered as indirect indicators/ predictors of DNA damage. Alterations in protein function caused by adducts might also be important in the disruption of cellular control which leads to cancer.	Detection of protein adducts - 3,4-Dihydroxy-l-phenylalanine as a biomarker of oxidative damage in proteins. Immunocomplex of enzyme (ICE) assay for detection of DNA–protein covalent complexes (DPCCs).
4. Clastogenic Effects	The disruption or breakages of chromosomes, leading to sections of the chromosome being deleted, added, or rearranged.	*In vivo or in vitro* Chromosomal aberrations Micronuclei Aneuploidy Abnormal karyotype

Toxicological Endpoint	Definition	Description/Prototypical Assays
5. Gene mutation	A change in the normal nucleotide sequence of DNA within a cell. Mutations can be silent or produce alterations in mRNA leading to abnormal protein expression. They are usually caused by copying errors during DNA replication (often due to the presence of DNA adducts) or as result of DNA damage such as strance breaks which could not be repaired by DNA repair mechanisms. These mechanisms often lead to base substitution, insertion, or deletion of one or more base pairs. They can produce major chromosomal restructing (see clasotgenic effects). Mutations can occur in oncogenes (e.g. k-ras), tumor-suppressor genes (p53, Tsc, VHL) or genomic instability genes (e.g. DNA repair genes).	Reversions & forward mutations in micro-organisms. Mutations affecting oncogenes, tumour-suppressor genes, and other genes involved in cell-cycle control. *In vitro* 1. Ames assay (reversions) in *Salmonella typhimurium* and reversions and forward mutations in *E. coli* 2. Other non-mammalian species—e.g. yeast (*Saccharomyces, Aspergillus*) 3. Mammalian mutation assays in endogenous genes used as markers for mutation—e.g. *Tk* (including mouse lymphoma assay), Aprt, *Xprt*, glycophorin A, *Hprt* / animal, *HPRT* / human *In vivo* 4. Mammalian gene-mutation assays a. Transgenic rodent assays (MutaMouse, BigBlue rat or mouse) b. Rodent dominant lethal assay—embryonic death indicating mutation and chromosomal aberrations in male germ cells c. Mouse specific locus mutation assays—measure of germ-cell mutations in the male parent

Toxicological Endpoint	Definition	Description/Prototypical Assays
		identified by phenotypic changes in offspring d. Mouse spot test—somatic mutations in embryonic melanoblasts after transplacental exposure, identifiable in the offspring as spots of different colour in the coat 5. Mutation assays in non-mammalian species a. Plants (*Tradescantia, Allium, Vicia*) b. *Drosophila melanogaster* i. Sex-linked recessive lethal assay—identifies heritable mutations in offspring ii. Somatic Mutation and Recombination Test [SMART] assay—identifies somatic mutations in wing cells (wing-spot test) and eye cells (eye-mosaic assay system)
6. Epigenetics	Epigenetics is the study of cellular and physiological traits that are heritable by daughter cells and not caused by changes in the DNA sequence; Epigenetics describes the study of stable, long-term alterations in the transcriptional potential of a cell. These effects can be caused by factors such as altered	DNA methylation, histone modification; alterations in miRNA expression in relevant genes

Toxicological Endpoint	Definition	Description/Prototypical Assays
	methylation of DNA, miRNA expression, changes in chromatin and histone structure.	
7. Changes in gene expression	This end point refers to alterations in the expression levels of gene active in the cell cycle and related facets of cellular function. These frequently arise through the promotion of epigenetic effects. But, they can also arise through a direct effect of the agent of through alterations in intracellular signalling, etc.	Alterations in mRNA or miRNA expression in relevant genes, pathways. Epigenetic changes in genomic instability genes (DNA replication and repair genes).
8. Alterations in cell signalling pathways	The ability of the agent to interfere with cell signaling pathways leading to expression of carcinogenic trait/phenotype in the cell e.g. facilitating cell invasion or induction of gene promotion for inflammatory mediators, oncogenes.	Alterations in cell signaling pathways (such as ras pathway, COX-2 pathway. togen-activated protein kinase (MAPK) pathway, ATM-p53,
9. Metabolites (reactive)	The agent under study is not itself reactive with DNA or other key cellular components. Instead, it required requires biotransformation (metabolic activation) by enzymes in organs such as the liver to produce active metabolites that are usually electrophilic.	Examples of metabolic activation include: - Formation of an alkylating agent – Oxidation to epoxide metabolites - Formation of arylnitrenium ion - These can often be identified through metabolism studies involving the analysis of tissues and fluids and often indicated by a

Toxicological Endpoint	Definition	Description/Prototypical Assays
		positive result in mutagenesis assays that only occurs in the presence of liver extract
10. Susceptibility	Susceptibility refers to individual variation in risk of developing cancer. This can arise from a range of factors including the presence of one or more inherited gene mutations (often marked by a family history that indicates an increased risk of disease) or exposures early in life (i.e. transplacental or in utero, early postnatal, lactational) (Anderson et al 2000).	genotype [vulnerability or genetic predisposition] developmental stage [life stage] Can be measured in vivo by SNPs, detection of genetic polymorphism in critical genes.
11. Immune effects	The immune system is a key factor in the response of the body to external agents, particularly viral, bacterial and parasitic organisms, but adverse effects on the functioning of the immune system can also result from exposure to chemical substances The immune system plays a major role in the inflammatory response to injury.. Altered immune function may lead to the increased incidence or severity of infectious diseases or cancer, since the immune system's ability to respond adequately to invading agents is suppressed. The inflammatory response to an agent can release cytokines and other factors that contribute to carcinogenesis.	Measures of altered function of the immune system that may lead to increased cancer risk (e.g., HIV-related effects) The mouse splenocyte assay.
12. [chronic] inflammation	Many cancers arise from sites of chronic inflammation. The tumour microenvironment, which is largely orchestrated by inflammatory cells, is an indispensable participant in the	Chronic inflammation leading to oxidative DNA damage. Assays

Toxicological Endpoint	Definition	Description/Prototypical Assays
	neoplastic process, fostering proliferation, survival and migration. In addition, tumour cells have co-opted some of the signalling molecules of the innate immune system, such as selectins, chemokines and their receptors for invasion, migration and metastasis.	Light microscopy, cytokine assays, and gene-expression profiles.
13. Cell death	Programmed cell death (apoptosis) is one of mechanisms by which a cell protects itself from DNA damage. In the presence of severe damage, the cell initiates a cascade that leads to the destruction of the cell. Other signals can also trigger this effect. *P53* plays a major role in the integrity of this process. Defects in programmed cell death can cause cancer (Adams, 1988). Evasion of apoptosis is a requirement for both neoplastic transformation and sustained growth of cancer cells (Hanahan and Weinberg, 2000; Weinberg, 2007).	Detection of cell death, including both inhibition and induction of apoptosis, autophagy, and necrosis Assays: 1. Apoptosis-specific assays: a. TUNEL (TdT-mediated dUTP Nick-End Labeling) assay. b. ISEL (*in situ* end labeling). c. DNA-ladder analysis for detection of DNA fragmentation d. Annexin-V analysis (membrane integrity). e. Detection of apoptosis-related proteins (p53, Fas, Bcl-2/Bax ratio, cytochrome c, caspases). f. Light-microscopic evidence of apoptotic nuclei. 2. Cytotoxicity and cell viability assays a. Clonogenic cell survival b. Trypan-blue or propidium-iodide exclusion (membrane integrity)

Toxicological Endpoint	Definition	Description/Prototypical Assays
		c. Cell-suspension counts using haemocytometer (manual) or Coulter counter (automated) d. Lactate-dehydrogenase (LDH) assay (membrane integrity) e. MTT (a tetrazolium dye) and related tetrazolium salts [MTS, XTT, or WSTs (water-soluble tetrazolium salts)]—colorimetric assays f. ATP-based bioluminescence assays g. Sulforhodamine B (SRB) assay h. Light-microscopic evidence of necrotic nuclei i. Light-microscopic evidence of missing cells in solid tissues j. Failure of appropriate background growth (Ames for cytostasis) or altered growth (e.g., decreased numbers of nuclei present for scoring in SCE assays).
14. [chronic] irritation	Chronic irritation can arise from a variety of external factors such as repetitive trauma and, exposure to acid (e.g. gastric acids). These factors create an environment of chronic inflammation which contributes to cancer (see end point #12: chronic inflammation).	Chronic irritation leading to chronic inflammation.

Toxicological Endpoint	Definition	Description/Prototypical Assays
15. Cell-cycle effects	Cellular replication is controlled by a very complex network of agents which regulate the cell cycle of division. These agents are responsible for preventing cell division in the presence of unrepaired DNA damage. Cell-cycle effects refer to an alteration of the functioning of this complex series of signalling pathways.	Detection of alterations in cell proliferation and cell-cycle effects (e.g., DNA replication changes, cell-cycle control, ploidy), mitogenesis *Assays:* 1. Replicative DNA synthesis (RDS) 2. BrdU labeling. 3. Proliferating cell nuclear antigen (PCNA) labeling. 4. Light-microscopic evidence of hyperplasia (e.g., thickening of epithelium). 5. Light-microscopic evidence of bi-nucleate cells. 6. Flow cytometry.
16. DNA-repair alteration	Cells contain multiple mechanisms to preserve genome integrity. These can involving repairing DNA which is damaged from adducts strand breaks, etc. Key mechanisms include: base excision repair (BER) and nucleotide excision repair (NER). Inherited abnormalities in DNA repair function lead to enhanced cancer susceptibility.	A. Inhibition of DNA-repair enzyme production or activity, loss of fidelity. B. Induction of DNA repair or transition from one repair pathway to another. C. mutations in DNA repair enzymes.
17. Receptor-mediated effects (pathway, gender specific, receptor-associated effects)	Receptor mediated effects are those that result when an extracellular signalling molecule activates a specific receptor located on the cell surface or inside the cell. In turn, this receptor triggers a biochemical chain of events inside the	Assays for Agonists binding of Ah receptor, estrogen receptor, androgen receptor and activation of the downstream signaling pathways and/or induction of the biological effect of the estrogens and androgens.

Toxicological Endpoint	Definition	Description/Prototypical Assays
	cell, creating a response. Depending on the cell, the response alters the cell's metabolism, shape, gene expression, or ability to divide.	
18. Hormonal effects	Hormones are chemicals secreted by the body and which control the homeostasis, reproduction, development, and/or behavior of local or remote tissues (e.g. insulin and the control of glucose metabolism). External agents can interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body. They can also demonstrate reactivity which is similar to endogenously produced hormones, which can lead to changes in homeostasis, reproduction, development, and/or behaviour.	Endocrine profiles; mammographic density measurement; ovariectomized animal model.
19. Angiogenic effects	Angiogenesis refers to the process of inducing blood vessel growth. This is a normal physiological function which is essential for growth and maintenance of organs and body tissue. Tumor growth requires the induction of new blood vessel growth in the tumor through the secretion of various growth factors (e.g. VEGF). This is commonly considered to be a hallmark of a tumor rather than of an exogenous agent.	Change in pro-angiogenesis factors, such as basic fibroblast growth factor (b-FGF). Neovascularization of tumor tissues in treated animals.

Toxicological Endpoint	Definition	Description/Prototypical Assays
20. Alterations in telomere length	Telomeres occur at the end of human chromosomes and consist of repetitive DNA sequences which facilitate replication of the ends of chromosomes. However, during each cycle of DNA replication, the length of telomere is reduced. Eventually, the reduction in length leads to cellular senescence. Carcinogenesis involves activation of a telomerase that prevents loss of telomere length, leading to immortalization of cells.	Reverse transcriptase-polymerase chain reaction analysis of extracted RNA to measure expression levels of the telomerase components.
21. Inhibition of gap-junctional intercellular communication	Gap junctions are plaque-like features on the cell plasma membrane. Complexes from adjacent cells can physical combine, providing a channel through which electronic signals and various signalling molecules (e.g. ions, second messengers, and low molecular weight metabolites) can pass from the interior of one cell to the other. This facilitates coordination of cellular metabolism and maintenance of homeostasis. Disruption of these communication pathways can cause a loss of 'contact inhibition' and abnormal cell growth.	A. Oncogenic transformation, i.e., anchorage-independent growth, loss of contact inhibition. *Assays:* Syrian hamster embryo cells. Cultured mouse fibroblast cells. Gap-junctional intercellular communication (GJIC): inhibition of metabolic cooperation or of dye transfer. Increased motility and invasiveness of cancer cell lines.
22. Bystander effects	The bystander effect was first identified in radiobiology and refers to the situation where non-irradiated cells exhibit effects caused by radiation as a result of chemical signals (messengers) received from nearby irradiated	

Toxicological Endpoint	Definition	Description/Prototypical Assays
	cells. These effects are often mediated through gap-junction transfer of chemical agents.	
23. Immortalization	In cell culture, normal cells have a fixed number of replication cycles before they enter cellular senescence and stop replicating. Immortalization refers to a stage where the cell can evade normal cellular senescence and will proliferate indefinitely. It is frequently associated with activation of telomerase (see end point #20).	Oncogenic transformation, i.e., anchorage-independent growth, loss of contact inhibition. *Assays:* 1. Syrian hamster embryo cells. 2. Cultured mouse fibroblast cells. 3. Gap-junctional intercellular communication (GJIC): inhibition of metabolic cooperation or of dye transfer. 1. Increased motility and invasiveness of cancer cell lines. 2. Cell transformation using Syrian hamster embryo cells.
24. Absorption, distribution, metabolism, excretion (ADME)	Evidence for the absorption, distribution, metabolism and elimination (ADME) of the agent affecting its carcinogenicity.	Pharmacokinetics, / toxicokinetics (PK/TK), mass balance studies, quantitative tissue distribution studies, metabolic profiling and identification.

Table 2. Ten key Characteristics of Human Cancer and Prototypical Agents Demonstrating these Characteristics

Characteristic	Prototypical Agents
1. Are electrophilic/or undergo metabolic activation [as a consequence, are chemically reactive in vivo]	Busulfan, Benzo[a]pyrene, Aflatoxins, tobacco smoke, Chlorambucil, Methyl-CCNU.
2. Produce Genotoxicity	Benzo[a]pyrene, 4-aminobiphenyl, cyclophosphamide, tobacco smoke.
3. Altered DNA repair and genomic instability	Beryllium, cadmium, nickel, Epstein Barr virus, Human Papilloma Virus, arsenic, Etoposide + cisplatin & bleomycin.
4. Epigenetic	Diethylstillbesterol, Hepatitis B virus, Hepatitis C virus, Human T-Lymphotrophic virus, asbestos, ionizing radiation, alcohol, Benzo[a]pyrene, coke production.
5. Oxidative stress	Ciclosporin, Hepatitis B virus, Schistosome Haematobioum, beryllium, Betel quid, dioxin.
6. Chronic Inflammation	Helicobactor pylori, Silica, asbestos fibers, Epstein Barr virus, Hepatitis viruses.
7. Immunosuppression	Cyclophosphamide, ciclosporine, azathioprine, Human immunodeficiency virus, Epstein Barr virus.
8. Receptor-mediated effects	Tamoxifen, estrogen-only menopausal therapy, combined estrogen oral contraceptives, dioxin, benzo[a]pyrene.
9. Immortalization	Human immunodeficiency virus, human papilloma virus, Human T-lymphotropic virus, Epstein Barr virus, Kaposi Sarcoma Herpes Virus, Hepatitis viruses.
10. Altered cell proliferation and death	Tamoxifen, diethylstilbestrol, Kaposi Sarcoma Herpes Virus, chronic infection with Clonorchis sinensis, benzidine.

Table 3: Linkage between the 24 Toxicological Endpoints and the 10 Key Characteristics of Carcinogenesis

Key Characteristic	Toxicological Endpoints	Description
1. Is electrophilic or can be metabolically activated to electrophiles	9. Metabolites (reactive)	Requires biotransformation (metabolic activation) produce reactive metabolites e.g. alkylating agents,epoxide metabolites, arylnitrenium ion.
	3. protein adducts	Formation of protein adducts that indicate of the presence of reactive metabolites, they are sometimes also considered as indirect indicators/ predictors of DNA damage (see 2 below)].
	24. Absorption, distribution, metabolism, excretion (ADME)	Evidence for the absorption, distribution, metabolism and elimination (ADME) of the agent affecting its carcinogenicity.
2. Is genotoxic	1. DNA damage	A. Direct Evidence of DNA damage—this category includes nuclear and mitochondrial DNA damage (*in vitro* or *in vivo*): DNA adducts DNA strand-breaks 1. DNA strand-breaks (single- and/or double-strand breaks). DNA-protein cross-links; DNA-DNA cross-links. B. Indirect indicators or biomarkers of DNA damage (*in vitro* or *in vivo*).
	4. Clastogenic Effects	Disruption or breakages of chromosomes leading to sections of the chromosome being deleted, added, or rearranged.

Key Characteristic	Toxicological Endpoints	Description
	5. Gene mutation	Reversions & forward mutations in micro-organisms. Mutations affecting oncogenes, tumour-suppressor genes, and other genes involved in cell-cycle control.
3. Alter DNA repair or causes genomic instability	16. DNA-repair alteration	Effects on key DNA-repair mechanisms include: base excision repair (BER) and nucleotide excision repair (NER). Inherited abnormalities in DNA repair function lead to enhanced cancer susceptibility.
4. Induces epigenetic alterations	6. Epigenetics	Stable, long-term alterations in the transcriptional potential of a cell. These effects can be caused by factors such as altered methylation of DNA, miRNA expression, changes in chromatin and histone structure.
5. Induces oxidative stress	2. Oxidative stress	Disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidant defenses within a cell.
6. Induces chronic inflammation	12. (chronic) inflammation 14. (chronic] irritation (leading to chronic inflammation)	Chronic inflammation and/or irritation leading to oxidative DNA damage.
7. Is Immunosuppressive	11. Immune effects	Measures of altered function of the immune system that may lead to increased cancer risk (e.g., HIV-related effects).
8. Modulates receptor-mediated effects	17. Receptor-mediated effects 18. Hormonal effects	Interference with cell signaling pathways leading to expression of carcinogenic trait/phenotype in the cell e.g. facilitating cell invasion or induction of gene promotion for inflammatory mediators, oncogenes. Interference with the synthesis, secretion, transport, binding, action,

Key Characteristic	Toxicological Endpoints	Description
		or elimination of natural hormones in the body. External agents can interfere with the synthesis, secretion, transport, Binding, action, or elimination of natural hormones in the body.
9. Cause immortalization	23. Immortalization	*A.* Oncogenic transformation, i.e., anchorage-independent growth, loss of contact inhibition. B. Increased motility and invasiveness of cancer cell lines C. Cell transformation.
	20. Alterations in telomere length	Activation of a telomerase that prevents loss of telomere length, leading to immortalization of cells.
10. Alters cell proliferation, cell death, or nutrient supply	15. Cell-cycle effects	Detection of alterations in cell proliferation and cell-cycle effects (e.g., DNA replication changes, cell-cycle control, ploidy), mitogenesis. Altered cell nutrient supply effects cell viability.
	22. bystander effects	The bystander effect was first identified in radiobiology and refers to the situation where non-irradiated cells exhibit effects caused by radiation as a result of chemical signals (messengers) received from nearby irradiated cells. These effects are often mediated through gap-junction transfer of chemical agents.
	21. inhibition of gap-junctional intercellular communication	Disruption of gap-junction intercellular communication pathways that can cause a loss of 'contact inhibition' and abnormal & anchorage-independent cell growth.
	19. Angiogenic effects	Change in pro-angiogenesis factors.

Key Characteristic	Toxicological Endpoints	Description
	13. Cell death	Induced defects in programmed cell death (apoptosis). Evasion of apoptosis is a requirement for both neoplastic transformation and sustained growth of cancer cells.
	8. alterations in cell signalling pathways	Interference with cell signaling pathways leading to expression of carcinogenic trait/phenotype in the cell e.g. facilitating cell invasion or induction of gene promotion for inflammatory mediators, oncogenes.

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